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TITLE: Does the Phenotyping of Disseminated Prostate Cancer
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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Approximately 20% of men who undergo a radical prostatectomy later relapse with bone metastases. The cellular events that are predictive of subsequent progressive disease remain unknown. We've focused attention on the detection of disseminated CaP cells in the blood and bone marrow. Our hypothesis is that these disseminated cells may provide critical insight regarding biomarkers of use in prognostication. Enrichment and isolation methods developed over the past three years by us allows for the identification and study of these cells in enriched populations or as individual cells. Using these combined new technologies we've begun to phenotype the disseminated CaP cells in blood and bone marrow of fifty patients undergoing a radical prostatectomy and who are found to have a Gleason Sum of ≥ 7 . Cells are also being stored and upon PSA biochemical progression, a subset will be analyzed so that comparisons of pre-progression and post-progression phenotypes can be made on the same patient and contrasted to (a) the phenotypic profiles of his primary tumor and (b) a disseminated CaP set of cells isolated from the bone marrow of patients with advanced disease following radical prostatectomy. Unfortunately, enrollment of patients was delayed by nine months due to DoD human subject issues.				
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INTRODUCTION

One of the great challenges in the clinical management of prostate cancer (CaP) is determining the risk of progression in those patients thought to have localized disease. The recurrence rate among men who elect a radical prostatectomy for presumed localized disease ranges from 15 to 25%. Historically, two approaches have been taken to detect disseminated cancer cells and to improve the "staging" of patients at the time of diagnosis. In the first, microscopic scrutiny of disseminated cells in the blood of cancer patients by immunohistochemistry (IHC) and cytogenetic techniques revealed important information regarding the features of these disseminated cells in general (1-7). On a parallel path were efforts employing molecular technology. For example, ten years ago we (8) and Moreno (9) were the first to propose using the molecular technique of reverse-transcriptase-polymerase-chain-reaction (RT-PCR) to "molecularly stage" patients at diagnosis in hopes that detection of disseminated PSA+ cells in blood or bone marrow (BM) would be predictive of recurrence. Over time, efforts in this field showed that PSA RT-PCR positivity was not highly correlative with recurrence. However, we postulated that enrichment of the presumed CaP cells in the BM aspirate prior to RT-PCR testing might reveal a high pre-surgical detection rate and that techniques might then be developed to isolate the individual disseminated cells for study. We made use of magnetic particle cell enrichment techniques to study disseminated cells in CaP patients (10, 11). This approach provides for a much more robust analysis of the disseminated cell population, especially since the yield from BM aspirates can be several hundred to thousands of cells of interest. One of the most striking revelations in our series has been the documentation that ~55% of CaP patients prior to radical prostatectomy have disseminated cells in their BM aspirates following enrichment. The Objectives of this proposal center upon the study of these disseminated cells recovered from blood and BM and whether their biological features overall or as individual cells are predictors for progression. It appears logical that residual disseminated CaP cells in the BM (~90% of patients with advanced disease have bone metastases (12)) following a radical prostatectomy should provide at least as much insight as those associated with the primary tumor that is removed at surgery. The enriched population is adequate for microscopic analysis or for isolation of individual cells for further study. A powerful component of our proposed analyses involves cDNA micro-array-gene chips developed by our colleagues Leroy Hood and Peter Nelson. This technique combines the proven chemistry of nucleic acid hybridization with advanced automation and image analysis to quantitatively assess gene expression profiles (13-15). The expression levels of thousands to tens of thousands of genes represented at 0.01-0.001% abundance in a population can be simultaneously assessed. Thus our ongoing studies focus on generating molecular and IHC phenotypes of disseminated BM cells in fifty patients who are undergoing radical prostatectomy and having a Gleason Sum of 7 or greater. These profiles are contrasted to those of the primary tumor and to those in patients who have relapsed with bone metastases. A unique aspect of this proposal is our profiling of not only pools of disseminated cells but individual cells as the technologies advance to this level of sensitivity. Furthermore, comparisons of profiles will be made between these patient populations and a subset that demonstrates PSA biochemical relapse. These studies will provide the first multiparameter phenotypic analysis/discovery of potential progression markers that takes advantage of advances (a) in the recovery of disseminated CaP cells from blood and bone marrow, (b) in technology allowing the isolation of viable CaP cells to provide three degrees of heterogeneity (enriched, pooled homogeneous and individual cells) and (c) in the fields of cDNA microarrays and informatics that target small cell numbers

BODY

Hypothesis:

Disseminated CaP cells isolated from the blood and BM at the time of radical prostatectomy will reveal biological features useful in assessing the probability of relapse. Furthermore, the analysis of single cells isolated from the enriched population will provide a second level of discrimination reflective of the heterogeneity of disseminated cells and allow detection of rare, but important, features not revealed in pooled, groups of disseminated cells.

Technical Objectives:

Task #1: Define the predominant phenotype(s) of prostate cancer bone metastases

- Identify 10 patients with advanced prostate cancer involving the bone who have had a radical prostatectomy and were found to have a Gleason Sum ≥ 7 . Following informed consent, obtain bone marrow aspirates. Using our paramagnetic enrichment techniques, we will derive an enriched population of disseminated CaP cells. From this population, we will "pluck" individual CaP cells and pool into sets of 50-100. These are then phenotyped using RT-PCR, PCR, FISH, ISH and micro-arrays (micro-array core facility of Peter Nelson, proposal co-investigator).
(0 – 12 months)

Task #2: Establish phenotype(s) of primary tumor, and disseminated cells in blood and bone marrow by enrichment and pooling of cells from 50 patients undergoing prostatectomy and having Gleason Sum ≥ 7 .

- Under separate funding we routinely obtain blood and bone marrow aspirates from all consenting patients prior to radical prostatectomy. From this large population this proposal involves a subset that consists of patients who are found to have a Gleason Sum ≥ 7 . We will select 50 of these patients for the studies herein. Using our para-magnetic enrichment and isolation techniques, we will derive populations of disseminated CaP cells in three degrees of "purity": (a) enriched, (b) isolated individual cells that are pooled (50-100 cells) and (c) individual cells (20+). Using the phenotyping protocols of Task #1, sets (a) and (b) will be phenotyped from blood and from bone marrow. Set (c) will be partially purified and stored at -80°C for use in Task #3. We will also obtain at least 2 primary tumor foci (microdissected) for phenotyping.
(0 – 24 months)

Task #3: Establish the phenotype of individual disseminated cells following enrichment and isolation from the bone marrow in patients of Aim #2 who experience biochemical (PSA) failure.

- Among the cohort of 50 patients, monitor for PSA biochemical recurrence using our ultrasensitive PSA chemiluminescent assay with which we've reported confidence in detecting relapse at a PSA serum level of 0.05 ng/mL.
(0-30 months)

- Identify the first 10 patients who experience a biochemical recurrence and 10 who do not show any evidence of recurrence. Retrieve from -80°C storage the individual aliquots of disseminated CaP cells (bone marrow derived only) that were processed in Task #2. Phenotype, primarily by micro-array, these 400 individual CaP cells (20 cells/patient x 20 patients [10 fail + 10 no fail]). (20 – 36 months)
- Compare and contrast all data sets, perform statistical analyses. (30 – 36 months)

Results:

We were extremely frustrated during year one because of the administratively imposed nine month delay in enrolling patients into the study. Although funding was awarded, a hold was placed on patient enrollment because of a disagreement between the University of Washington IRB and the DoD over language in the consent form. The primary issue was the initial insistence that all enrolled patients have identifying personal data entered into a national DoD database. This issue took nine months to resolve with the DoD finally removing this requirement as part of new policy. Unfortunately this delay, which was initially anticipated to be resolved in weeks, resulted in our reassignment of some personnel initially designated to work on this proposal to other funded projects and the reduction in effort by other personnel. In addition, the sub-contract between the University of Washington and Peter Nelson, MD. of the Fred Hutchinson Cancer Research Center was not implemented to save costs as there were no patient specimens for cDNA micro-array analysis. As briefly detailed below, an effort was made during this period to fine tune the cell isolation techniques and those involving dual staining of cells on a cytospin slide using excess blood or bone marrow specimens from other funded studies in the laboratory. Once we received permission to begin the patient studies we as quickly as possible geared up to enroll patients and began the paperwork to establish the FHCRC subcontract. Yet, these efforts took a couple of months so in fact we lost nearly 11 of our first 12 months. In looking over our projections, it is possible that we can catch up but we most likely will ask for a one-year, no cost, extension once we see our patient accrual status in year 3.

Development and Refinement of Critical Techniques:

During the period from the time this proposal was submitted and month 9 of year 1, three other techniques became available for the enrichment of disseminated cancer cells: Dynal, Stem Cell Technologies and Oncopure. In the first several months of year 1 we compared these three methods to the Miltenyi method we had developed and utilized in generating the preliminary data for this proposal. In all instances the Miltenyi method proved superior to these other methods because of its versatility in allowing us to retrieve isolated, viable disseminated cells from the enriched population. We found the Oncopure method to be quicker (16) and the Dynal system to yield a more pure enriched population but neither provided the required versatility of the Miltenyi system for the subsequent isolation and study of the disseminated cancer cells.

Although we had reduced staff, we devoted a consider effort in developing and refining three phenotyping techniques that would be applied to the patient specimens. The first challenge was to accurately identify the percent of proliferating (Ki-67 positive) cells among the disseminated epithelial (Cytokeratin, CK- positive) cells. This required a dual immunostain on a

single cytopsin specimen of enriched cells. We had obtained good immunostaining when separate cytopsin slides were used for the Ki-67 and CK analysis but ideally this would be done on a single cytopsin slide. However we soon discovered that we could not simply combine the procedures when dual staining was required on a single slide. This is because Ki-67 works best when antigen retrieval is done, but by doing so, there is no CK stain. Numerous trial and error attempts finally led to a procedure, that while a bit cumbersome, allows the detection of both markers on a single cytopsin slide.

Fluorescent in situ hybridization (FISH) was the third phenotypic procedure that was developed. This would enable us to detect chromosomal aberrations on CK positive circulating tumor cells. Initially we prepared test specimens consisting of spiked LNCaP cells into normal peripheral blood buffy coat. As stated in the protocol, we obtained the help of Barbara Trask, Ph.D. of the FHCRC who has had extensive expertise in FISH. However, she had not previously attempted to perform FISH on disseminated tumor cells. A CEP 8 Spectrum Orange direct label chromosome enumeration DNA Probe kit (Vysis) was used for the FISH learning procedure and the procedure was performed according to kit directions, except hybridization was at done 37° C and incubated overnight. We found that acetone fixation was superior to 4% formalin fixation. The LNCaP cells had 3 spectrum orange positive areas per cell (indicating 3 chromosome 8), while the normal WBC had 2 as expected. We were able to reproduce these results in our lab (Figure 1). Next, as in the CK and Ki-67 analysis, we needed to develop a technique for the simultaneous identification of CK+ cells and within those cells to assess chromosomal aberrations by FISH. Three methods were found in the literature but none proved to be highly reproducible when using the spiked LNCaP cells. Multiple combinations of immunostaining substrates and fluorescent chromagens were then compared as we modified the given procedures. Several of these studies were performed in the Trask lab. Unfortunately, the combination that proved best required a filter set not available on our fluorescent scope. Therefore, using funds outside this proposal, we've ordered a new inverted fluorescent microscope that will have the objectives, filters, and digital camera needed for FISH analysis with multiple flourophore labels.

KEY RESEARCH ACCOMPLISHMENTS

- During the prolonged delay in enrolling patients we expended our efforts toward the development of techniques critical for the simultaneous detection of the proliferation marker Ki-67 among CK+ epithelial cells and the simultaneous detection of chromosomal aberrations by FISH among CK+ epithelial cells.
- We compared three competing technologies for the enrichment of disseminated cancer cells to the one we've developed, finding that ours was still preferred because of its versatility which allowed isolation and study of individual, viable cells from the enriched population.
- At approximately month 12 we began to accrue patients into the study.

REPORTABLE OUTCOMES

Abstract:

Pfitzenmaier J, Arfman E, Klein J, Winch R, Nance M, Lange P and Vessella R. New enrichment method for the isolation and characterization of circulating prostate cancer cells (CPCC) from the peripheral blood (PB). Proc. Am Assoc. Cancer Res 43:433 #3635

CONCLUSIONS

Our year 1 progress was extremely disappointing due to the nine month delay in receiving permission from the DoD to obtain patient specimens followed by a further couple of month "start-up" period. Nevertheless, significant efforts were expended in developing critical techniques that will be applied to the biological and molecular phenotyping of disseminated CaP cells in patients with advanced disease in the bone and those pre-radical prostatectomy. To our knowledge, we still remain the only group applying the most advanced micro-array expression technologies with those of classical phenotyping and FISH analysis to the study of disseminated prostate cancer cells isolated from the bone marrow down to the single cell level.

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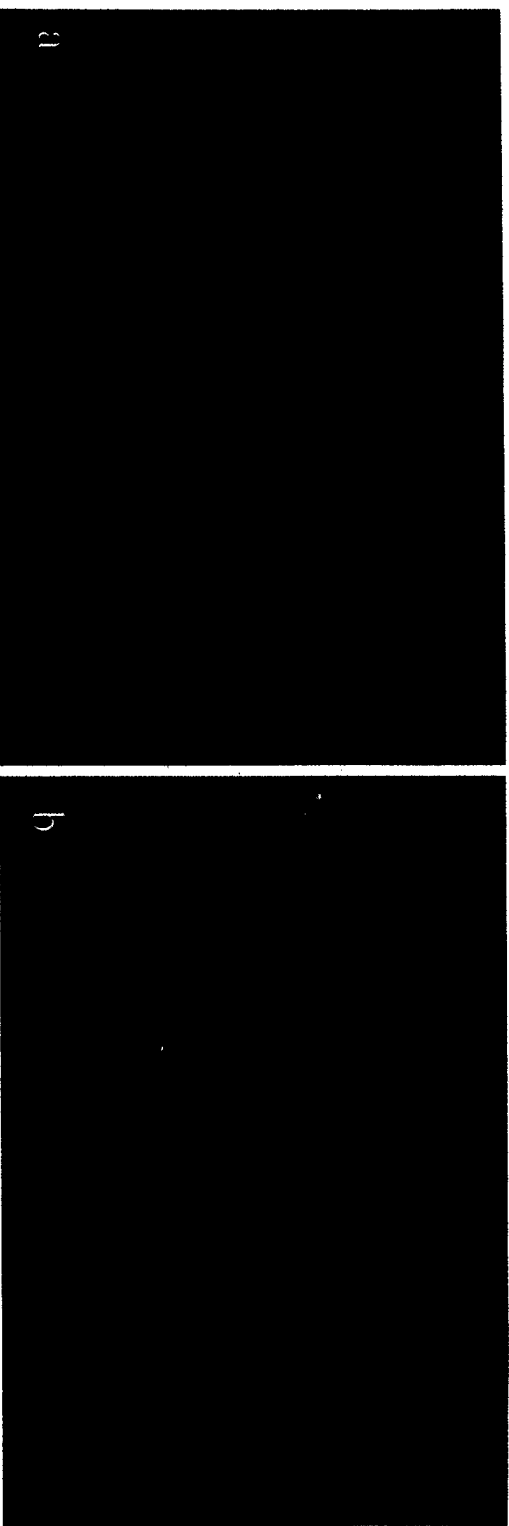


Figure 1: This figure shows fluorescence in situ hybridization (FISH) with a probe against the centromeric region of chromosome 8. DAPI (grey) was used to counterstain the nucleus. In a.) two white blood cells of a control person are shown (two red spots per cell indicating two centromeric regions of chromosome 8) whereas in b.) two LNCaP prostate cancer cells are shown with three centromeric regions in each of them